Protocol

Analyzing DNA Replication II: Fixation and Processing of Tissues and Cells Labeled with Bromodeoxyuridine (BrdU)

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This protocol was adapted from "Analyzing DNA Replication: Nonisotopic Labeling," Chapter 13, in *Basic Methods in Microscopy*, (eds. Spector and Goldman). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA, 2006.

INTRODUCTION

The number of cells traversing the cell cycle and the rate of progression through it provide important indices of cell growth and tumorigenicity. S-phase cells can also be identified by their high content of DNA polymerase and proliferating cell nuclear antigen, a component of the leading-strand polymerase. Although both these markers can be detected rapidly and conveniently using the appropriate antibodies, neither are found exclusively in S-phase cells. Immunolabeling after incorporation of modified DNA precursors (e.g., 5-bromodeoxyuridine [BrdU, bromodeoxyuridine]) allows more rapid and precise detection of cells in S-phase of the cell cycle. BrdU is phosphorylated by cells to give BrdUTP, and this precursor is incorporated into DNA instead of deoxythimidine triphosphate. In living cells, BrdU is incorporated into replication sites that can then be detected using fluorochrome or enzyme-coupled antibodies. Alternatively, DNA synthesis sites can be labeled at high resolution by incubating cells with analogs of the natural precursors of DNA. The cells are then fixed and the incorporation sites are detected using fluorochrome- or enzyme-tagged antibodies. Formaldehyde- or alcohol-based fixation and paraffin embedding are used frequently. Formaldehyde, a mild protein cross-linking reagent, preserves cell structure well, and washing with a nonionic detergent or organic solvent permeabilizes membranes and allows antibodies access to the interior of the cell. This protocol describe the methods necessary to fix and process tissue and cell samples for subsequent antibody detection of loaded DNA precursors.

RELATED INFORMATION

Procedures for incorporating DNA precursors into tissues in whole animals in vivo, isolated tissue in vitro, and in cultured cells are described in **Analyzing DNA Replication I: Labeling Animals, Tissues, and Cells with Bromodeoxyuridine (BrdU)** (Jackson and Cook 2008a). Methods for labeling samples fixed using the protocols described here are presented in **Analyzing DNA Replication III: Antibody Labeling of Incorporated Bromodeoxyuridine (BrdU) in Tissues and Cells** (Jackson and Cook 2008b).

MATERIALS

CAUTIONS AND RECIPES: Please see Appendices for appropriate handling of materials marked with <**!**>, and recipes for reagents marked with <**R**>.

Reagents

<**!**>Chloroform (for tissue fixation; see Step 6)
Ethanol (70%, 95%, and 100%) (for tissue fixation; see Step 7)

<R><!>Formaldehyde (4%, w/v in PB) (ice-cold)
<R><!>Formaldehyde (4%, w/v in PBS)

<R>*Carnoy's fixative for paraffin embedding can be used in place of formaldehyde in PBS in tissue fixation (see Step 1).*

Material of interest (e.g., isolated tissues, cultured cells, etc.), precursor-labeled as in **Analyzing DNA Replication I: Labeling Animals, Tissues, and Cells with Bromodeoxyuridine (BrdU)** (Jackson and Cook 2008a)
Paraffin
<R>Phosphate-buffered saline (PBS) (ice-cold for Step 18)
<R>Physiological buffer (PB) (for fixation of encapsulated cells; see Steps 14-17) (ice-cold for Steps 14 and 16)
<!>Triton X-100 (for fixation of cells grown on glass and encapsulated cells; see Steps 12 and 15)

Equipment

Embedding molds
<!>Microtome
Slide warmer preset to 60°C
Slide washing jars
Slides (glass)

METHOD

Tissue Fixation

Frozen sections can be used if sites of replication are to be labeled in conjunction with fixation-sensitive antigens.

1. Place precursor-labeled tissue sample in freshly prepared fixative (4% formaldehyde in PBS or Carnoy's fixative).
2. Incubate for 2-16 h at 20°C to allow the fixative to permeate the sample.
   Replace with fresh fixative once during the incubation.
3. Embed the sample in paraffin.
4. Using a microtome, cut 3-5-µm sections and collect on clean glass slides.
5. Incubate slides for 30 min at 60°C.
6. Dewax sections in two changes of chloroform, 3 min each.
7. Rehydrate the sections through a graded ethanol series:
   i. Two changes of 100% ethanol, 3 min each.
   ii. Two changes of 95% ethanol, 3 min each.
   iii. Two changes of 70% ethanol, 3 min each.
8. Rinse sections in H_2O.
   Samples are now ready for antibody labeling.
   See Troubleshooting.

Fixation of Cells Grown on Glass

Samples of soft or dispersed tissues, needle aspirates, or blood can be spread on slides, air-dried, fixed with organic fixatives, rinsed in PBS, and stained using this procedure. Such preparation does not preserve tissue architecture well but can be useful in preliminary screens.

9. Rinse samples in PBS.
10. Incubate coverslips or slides in 4% formaldehyde in PBS for 10 min at room temperature.

11. Wash coverslips or slides twice with PBS.
See Troubleshooting.

12. Prepare 0.2% (v/v) Triton X-100 in PBS. Permeabilize samples by incubation in this solution for 5 min at room temperature.

13. Wash the samples gently in three changes of PBS over a period of 5 min (total time).
Samples are now ready for antibody labeling.
See Troubleshooting.

Fixation of Encapsulated Cells

14. Remove unincorporated precursors by washing beads three times in 10 volumes of ice-cold PB, 5 min each.

15. Prepare 0.2% (v/v) Triton X-100 in PB. Permeabilize samples by incubation in this solution for 10 min at 0°C.

16. Wash the samples three times in 10 volumes of ice-cold PB, 5 min each.

17. Fix the samples in ice-cold 4% formaldehyde in PB for 10 min.

18. Wash the samples three times in 10 volumes of ice-cold PBS.
Samples are now ready for antibody labeling.
See Troubleshooting.

TROUBLESHOOTING

Problem: Cells detach from glass slides or coverslips.
[Step 11]
Solution: Try an organic fixative such as 90% ethanol in H_2O (which gives reasonable morphological preservation) for 10 min at room temperature prior to Step 9. Alternatively, try fixation in methanol or acetone.

Problem: There is high background.
[After Steps 8, 13, and 18]
Solution: This may be due to excess unincorporated precursor in the sample. Permeabilize the samples with Triton X-100 and wash with PBS (Steps 12 and 13) prior to fixation and washing (Steps 10 and 11).

DISCUSSION

Double labeling can be performed on cells grown in BrdU. This is useful to assess the cell cycle dependency of different antigens. Some modification of standard techniques (such as microwave treatment) may be required to optimize antigenicity (Connolly and Bogdanffy 1993). If single-cell suspensions are available, cells can be fixed directly in suspension and labeling indices can be assessed by flow cytometry (see, e.g., Dolbeare et al. 1983; Landberg and Roos 1991). This allows the labeling intensity of large populations to be determined but neglects morphological detail.

REFERENCES

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Cold Spring Harbor Protocols

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Cold Spring Harb Protoc; doi: 10.1101/pdb.prot5032